(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 January 2001 (04.01.2001)

PCT

(10) International Publication Number
WO 01/00237 A1

(51) International Patent Classification[7]: A61K 39/385, C12P 21/00, C07K 16/00, 17/00, G01N 33/48

(21) International Application Number: PCT/US00/17609

(22) International Filing Date: 27 June 2000 (27.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/141,699 30 June 1999 (30.06.1999) US

(71) Applicant: **SIGMA-ALDRICH CO.** [US/US]; 509 West Monroe Street, Highland, IL 62249 (US).

(72) Inventors: **GOLDFORD, Marc**; 704 Bitterfield Drive, Ballwin, MO 63011 (US). **TRISCOTT, Mark, X.**; 461 Governors Court, Ballwin, MO 63021 (US).

(74) Agents: **HEJLEK, Edward, J.** et al.; Senniger, Powers, Leavitt & Roedel, One Metropolitan Square, 16th Floor, St. Louis, MO 63102 (US).

(81) Designated States *(national)*: AU, CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

A6 - 09/819,136

(54) Title: SYNTHETIC PEPTIDE IMMUNOGENS AND ANTIBODIES THERETO

(57) Abstract: Provided are methods for selecting peptide immunogens for a target protein from a first species which has an amino acid sequence different from the analogous protein from a second species. The method comprises selecting peptide fragments from the target protein which are hydrophilic and which vary in sequence from the analogous protein by at least one amino acid. Antibodies made using the selected immunogens conjugated to a carrier molecule are also provided. Antibodies made by this method to rabbit tissue factor which do not cross-react with human tissue factor are also provided.

SYNTHETIC PEPTIDE IMMUNOGENS AND ANTIBODIES THERETO

FIELD OF THE INVENTION

The present invention generally relates to methods for selecting peptide immunogens to produce antibodies to a target protein which exhibits amino acid variation between species. More specifically, the present invention relates to methods of selecting peptide immunogens and producing species-specific antibodies to a mammalian tissue factor, and antibodies produced by those methods.

DESCRIPTION OF RELATED ART

Antibodies to protein antigens have been used for many years for numerous research and diagnostic purposes. Antibodies can be made to specific regions of a protein by injecting a small peptide portion of the protein (5-50 amino acids) which has been conjugated to an immunogenic carrier (such as keyhole limpet hemocyanin [KLH]). The immunized animal will recognize the peptide as an epitope of the immunogenic carrier. This approach has advantages over the more common methods involving the injection of the whole protein. For example, this approach allows antibody reagents to be made which bind to specific, predictable domains of the protein of interest. These anti-peptide antibodies are useful for epitope mapping in structure/function studies, immunohistochemical localization, and immunoaffinity purification of the protein of interest. One particular protein of interest for this invention is mammalian tissue factor.

Tissue factor (TF) is a transmembrane glycoprotein which plays a critical role in coagulation (blood clotting). Normal coagulation begins when vascular endothelium is damaged. Damaged vascular endothelium exposes TF to plasma, allowing TF to bind to factor VII, a serine protease zymogen. The binding of TF to factor VII, and subsequent activation to factor VIIa, is the primary step for coagulation initiation. Subsequent conformational changes and Ca2+ bridge formation allow the TF/FVIIa complex to bind to and to activate factor X. Activated

factor X converts prothrombin into its active serine protease, thrombin. Thrombin converts fibrinogen into fibrin. Finally, fibrin fibers associate with one another to form a clot, or a platelet-fibrin network that prevents further bleeding.

The amino acid sequence of TF protein between mice, rabbits, and humans is highly conserved. Pawashe et al., 1991, Thromb. Haemost. 66:315. The crystal structure of TF consists of two immunoglobulin-like domains with an extensive interdomain interface region which contains the binding site for factor VII. Harlos et al., 1994, Nature 370:662.

TF is expressed in various tissues such as cardiac myocytes, renal glomeruli, the granular layer of the epidermis, the epithelium of oropharynx and vagina, and intestinal, urinary bladder, respiratory mucosa, and at tissue barriers between the body and the environment. Ruf et al., 1994, FASEB J. 8:385. Tissue factor has also been implicated in the pathogenesis of various infectious, cardiovascular and neoplastic diseases. Excessive coagulation, for example, leads to stroke and ischemia, resulting in infarction of tissues. Acute myeloblastic leukemias are known to express TF, resulting in disseminated intravascular coagulation (DIC), a disorder characterized by excessive bleeding due to rapid consumption of platelets and disruption of fibrin polymerization. *Id.* A better understanding of TF, therefore, allows researchers to design more effective therapies for controlling coagulation. Such control can potentially alleviate complications in patients suffering from these and other diseases.

Several antibodies have been made to TF. Most of these anti-TF antibodies were made to the whole TF protein. For example, monoclonal antibodies to native, factor VII-affinity purified human TF were used to study the essential regions of the extracellular domain of TF. Magdolen et al., 1998, Biological Chemistry 379:157. Other antibodies have been made to purified or recombinant TF from humans and rabbits. See, e.g., Taylor et al., 1991, Circulatory Shock 33:127; Morissey et el., 1988, Thrombosis Res. 52:247; Pawashe et al., 1994, Circulation Res. 74:56; U.S. Pat. App. 5,223,427. However, membrane-bound

proteins such as TF are difficult to purify, especially in its native folding conformation. In another approach, monoclonal antibodies were made from mice injected with murine cultured cells expressing recombinant rabbit TF. Speidel et al., 1995, Circulation 92:3323.

Anti-peptide antibodies to TF has been made. In one case, polyclonal antibodies were made to human TF regions which allow them to inhibit TF binding to factor VII. Ruf et al., 1991, Biochem. J. 278:729. In another case, anti-peptide monoclonal antibodies were made to the C-terminal cytoplasmic domain of human TF. Carson et al., 1992, Blood Coagulation and Fibrinolysis 3:779.

Antibodies made to proteins such as TF which have extensive homology between species would not be expected to exhibit species-specificity. This has been shown to be the case with TF, where monoclonal antibodies to human tissue factor cross-react and inhibit baboon TF. Taylor et al., 1991, Circulation Shock 33:127. This could be a problem, e.g., where the antibodies are used to diagnose diseases and are required to distinguish between two pathogenic species. Proteins such as TF with extensive sequence homology among vertebrates are also limited in the range of antibodies they would be expected to induce. Since there is little sequence difference between the protein from the species of interest and the same protein in the immunizing animal, the immune system of the immunizing animal would recognize the homologous regions of the injected protein as a "self" epitope and would thus only produce antibodies to immunodominant regions of the injected protein which are not homologous with the injected animal's analogous protein. For example, murine monoclonal antibodies to whole human TF could only be produced to regions of the human TF which are dissimilar to mouse TF. If antibodies are desired to many regions of the TF protein, for example for structure-function studies, the results would likely be disappointing. Therefore, there is a need for improved methods for making antibodies to proteins which show extensive amino acid homology between species, where the antibodies need to

be species-specific or where antibodies are desired to several regions of vertebrate proteins.

SUMMARY OF THE INVENTION

Among the objects of the invention may be noted the provision of methods for determining peptides of a target protein for use as immunogens in order to reliably produce antibodies which are species-specific. A more specific object of the invention is the provision of methods for determining regions of tissue factor for use as immunogens to produce antibodies which are species-specific. Another object of the invention is the provision of antibodies to tissue factor made using the above methods.

Briefly, therefore, the present invention is directed to a method for selecting a peptide immunogen of a target protein from a first species, wherein the target protein comprises an amino acid sequence which varies between the first species and a second species. The method comprises (a) identifying peptide regions of the amino acid sequence of the target protein from the first species which has a hydrophilicity value greater than 0, wherein the regions are 5-50 amino acids in length, and (b) selecting a peptide immunogen from regions identified in step (a) which have at least 1 nonhomologous amino acid between the first species and the second species.

The present invention is also directed to a method of making an antibody which is specific for a target protein of a first species, wherein the target protein comprises an amino acid sequence which varies between the first species and the second species. The method comprises selecting a peptide immunogen of the target protein by the above-described method, synthesizing the peptide immunogen, conjugating the peptide immunogen to an immunogenic carrier molecule to make a peptide-carrier antigen, and producing antibodies to the antigen.

Additionally, the present invention is directed to an antibody made by the above-described method.

Furthermore, the present invention is directed to a peptide which consists of a sequence from a tissue factor extracellular

region from a first species which is 5-50 amino acids in length, has a hydrophilicity value greater than 0, and has at least 1 nonhomologous amino acid from a tissue factor from a second species.

The present invention is also directed to an antigen, and antibodies made to an antigen, where the antigen comprises an immunogenic carrier molecule conjugated to a peptide which consists of a sequence from a tissue factor extracellular region from a first species which is 5-50 amino acids in length, has a hydrophilicity value greater than 0, and has at least 1 nonhomologous amino acid from a tissue factor from a second species.

ABBREVIATIONS AND DEFINITIONS

To facilitate understanding of the invention, a number of terms as used herein are defined below:

As used herein, "TF" shall mean tissue factor.

As used herein, the term "species-specific", when used to describe an antibody, denotes that the antibody will bind to a protein of one species but not the same protein of another species. An antibody which binds to (or "reacts" or "recognizes") a protein from a first species but not the same protein from a second species is said to be species-specific to the protein from the first species, because it does not "cross-react" to the protein from the second species.

As used herein, the term "synthetic peptide" or "peptide" is a chain of 5 to 50 amino acids covalently linked to one another by peptide bonds.

As used herein, an "antigen" is a macromolecule to which antibodies are made. The antigen is usually injected into a vertebrate, which then elicits production of antibodies to regions ("epitopes") of the antigen.

As used herein, the term "immunogen" is a portion of an antigen to which antibodies are desired. An immunogen may be an epitope of the antigen or it may be comprised of several epitopes. More specifically, the term "immunogen" is applied herein to mean a peptide from a protein of interest which is

conjugated to an immunogenic carrier molecule for the purpose of eliciting antibodies to the immunogen portion of the antigen.

As used herein, the term "hydrophilic" when used in reference to amino acids refers to those amino acids which have polar and/or charged side chains. Hydrophilic amino acids include lysine, arginine, histidine, aspartate (i.e., aspartic acid), glutamate (i.e., glutamic acid), serine, threonine, cysteine, tyrosine, asparagine and glutamine. The terms "hydrophilicity value" or "Hydrophilicity index value" as used herein means a number value assigned to each amino acid from 3.0 to -3.4, where the larger numbers are more hydrophilic. The hydrophilicity values are calculated using the method of Hopp et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 76:3824. Also, a moving hydrophilicity average of these values for six amino acids have been calculated using the Hopp et al. method as described above.

As used herein, the term "hydrophobic" when used in reference to amino acids refers to those amino acids which have nonpolar side chains. Hydrophobic amino acids include valine, leucine, isoleucine, cysteine and methionine. Three hydrophobic amino acids have aromatic side chains. Accordingly, the term "aromatic" when used in reference to amino acids refers to the three aromatic hydrophobic amino acids phenylalanine, tyrosine and tryptophan.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a sequence homology comparison of the rabbit, human, and mouse tissue factor proteins. The figure was taken from Pawashe et al., 1991, Thromb. Haemost. 66:315.

FIGURE 2 is a list of candidate immunogenic peptides from rabbit tissue factor selected using the methods of the present invention.

FIGURE 3 is a list of candidate immunogenic peptides from human tissue factor selected using the methods of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The contents of each of the references cited herein are herein incorporated by reference. The procedures disclosed herein which involve the molecular manipulation of nucleic acids are known to those skilled in the art. *See generally* Fredrick M. Ausubel et al. (1995), "Short Protocols in Molecular Biology", John Wiley and Sons, and Joseph Sambrook et al. (1989), "Molecular Cloning, A Laboratory Manual", second ed., Cold Spring Harbor Laboratory Press, which are both incorporated by reference.

The present invention is directed to methods of selecting a peptide immunogen consisting of a region of a target protein from a first species, where the target protein has a different amino acid sequence in a second species. The immunogen is selected for the purpose of eliciting antibodies to the region of the target protein containing the peptide. The method comprises the following steps: (a) the amino acid sequence of the target protein is analyzed for hydrophilicity, and regions from 5-50 amino acids long are identified which are hydrophilic, for example having a value greater than 0 using the method of Hopp et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 76:3824; and (b) the hydrophilic amino acid regions of the target protein are compared to the same regions from the same protein of the second species, and a region is selected as the peptide immunogen which has at least 1 nonhomologous amino acid between the first species and the second species. Antibodies are then made to the selected immunogen after conjugating the immunogen to an immunogenic carrier molecule. Antibodies which are generated to the immunogen generally do not bind to the same protein of the second species.

The method of the current invention may be applied for the production of antibodies to any target protein in a species of interest to which there exists an analogous protein in another species which has an amino acid sequence which is different from the amino acid sequence in the target protein in the species of interest. Included are proteins from any prokaryotic, eukaryotic, or viral source, including but not limited to,

vertebrates, invertebrates, protozoans, bacteria, fungi, and plants.

The amino acid sequences of the target protein and the analogous protein from the second species can be obtained from various sources, such as published material, as well as protein sequencing (e.g. Edman degradation). The amino acid sequences may also be deduced from the genes encoding the target proteins which can be obtained from published sources or by cloning the genes encoding the target proteins from both species and determining the start codon by known methods.

The regions of the target protein which are hydrophilic are utilized because those regions tend to be more antigenic than hydrophobic regions. Hydrophilic peptide regions also tend to localize on the outer surface of the target protein, and are therefore more accessible for eliciting an antibody response and for being exposed to an antibody in *in situ* studies such as immunocytochemical investigations. Using hydrophilic peptides to produce immunogens also have the advantage of being more soluble in aqueous solution than hydrophobic peptides. This makes the procedure for conjugating the peptides to carrier proteins simpler with preferred conjugation procedures. Additionally, antigens comprising hydrophilic peptides are more soluble than antigens comprising hydrophobic peptides, making immunization procedures with the former antigen simpler.

A hydrophilicity analysis provides a determination of the moving average of the polar portion, preferably a hexapeptide, of the protein. The hydrophilic epitopes of tissue factor may be determined by the method of Hopp et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 76:3824. Computer software, such as MacVector from International Biotechnologies, Inc., are also available to analyze hydrophilicity of a target protein. Using the Hopp method where hydrophilicity values for individual amino acids range from -3.4 (for tryptophan) to 3.0 (for lysine, glutamic acid, aspartic acid, and arginine), the hydrophilicity is preferably determined by averaging the mean hexapeptide hydrophilicity value over the length of the peptide. Peptides

may be selected which have a hydrophilicity greater than 0. Preferred peptides have a hydrophilicity greater than 0.2.

After selecting hydrophilic peptides from the regions of interest in the target protein, the sequence of each peptide is compared with the analogous sequence in the same protein from the second species. To make this comparison, the amino acid sequences of the target protein and the same protein from the second species are preferably aligned by 3-dimensional structural similarity. The structural similarity between the two sequences can be determined by comparing predicted 3-dimensional structure which can be determined by a number of computer programs, or by determining the crystal structure of both of the proteins by known methods (see, e.g., Harlos et al., 1994, Nature 370:662 for the crystal structure of TF). However, 3-dimensional structural similarity can generally be ascertained by aligning the sequences of the two proteins by introducing gaps in one or the other sequence to minimize mismatches. This task can also be performed by a number of well-known computer programs (see, e.g., Altschul et al., 1997, Nucl. Acids Res. 25:3389). See Figure 1 for such an alignment of mouse, rabbit, and human TF.

The aligned sequences of the candidate hydrophilic peptides are compared for sequence homology. Peptides which have at least 1 nonhomologous amino acid between the target protein and the analogous protein from the second species may be used as immunogens according to the present invention. Preferably, the candidate hydrophilic peptides have at least 3 nonhomologous amino acids between the target protein and the analogous protein from the second species. The nonhomologous amino acids of the candidate hydrophilic peptides may or may not be consecutive in the peptide chain. Preferably, the homology between the two peptide sequences is less than 75%. Even more preferably the homology between the two peptide sequences is less than 60%. As the sequence homology between the two peptide sequences decreases, the species-specific properties of the antibodies generated from the immunogen of the protein of the first species increases, thereby decreasing the possibility that the antibody will cross-react with the same protein of the second species.

After the peptide immunogen is selected, it is synthesized in preparation for conjugation to the carrier molecule. Synthesis of the peptide can be by any convenient method, for example by translating a nucleic acid sequence encoding the peptide in a suitable recombinant host cell, e.g., *E. coli*, then purifying the peptide by routine methods (e.g., reversed phase HPLC). However, the peptide is preferably synthesized using chemical methods, preferably solid phase methods. The preferred method for this employs Fmoc chemistry (Fields et al., 1990, Int. J. Pept. Protein Res. 35:161; Wellings et al., 1997, Meth. Enzymol. 289:44). Preferably, the peptide is selected with a terminal amino acid residue suitable for coupling to the carrier with a crosslinking reagent, or synthesized with an additional amino acid residue at the terminus for this purpose. Examples of such amino acids are tyrosine, for a bis-diazobenzidine (BDB)-activated carrier; aspartic acid, glutamic acid, or a free carboxy terminus, for a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)-activated carrier; or cysteine, lysine, or a free amino terminus, for a m-maleimidobenzoyl-N-hydrosuccinamide (MBS)-activated carrier. Preferred is cysteine and MBS carrier activation.

After the peptide immunogen is synthesized, it is conjugated to a carrier molecule. The carrier is preferably an antigenic, soluble protein. Preferably, the carrier is selected which would not elicit antibodies which would bind to antigens in the first or second species. A preferred carrier is keyhole limpet hemocyanin (KLH).

Alternatively, the peptide immunogen can be conjugated to a resin-polylysine carrier, to follow the multiple antigenic peptide strategy. See, e.g., McClean et al.,1991, J. Immunol. Meth. 137:149.

Production of the antigen is achieved by conjugating the peptide immunogen to the carrier molecule. This conjugation preferably proceeds by activation of the carrier, e.g., by BDB, EDC, or, preferably, MBS, then addition of the peptide.

The antigen is then used to produce an antibody which binds to the immunogen. The antibody may be a mono-specific antibody.

The monospecific antibody may be a monoclonal antibody produced, for example, by the method of Galfre et al., 1977, Nature 266:550. Alternatively, the monospecific antibody may be a recombinant antibody produced, for example, by the method of Lowman et al., 1991, Biochemistry 30:10832.

The antibody can also be a polyclonal antibody. The polyclonal antibody can be prepared, for example, by immunizing a mammal such as a mouse, goat, sheep, or rabbit with the peptide-carrier antigen and subsequently isolating the serum therefrom to obtain an antiserum that contains the polyclonal antibodies.

The antibody is preferably characterized by determining its ability to bind to the immunogen and the target protein by, e.g., ELISA, hemagglutination, fluorescent antibody binding to tissue, western blot, or any other suitable procedure. Tissue factor antibodies made by the invention method can be tested, e.g., for reactivity to the immunogen, and to recombinant and/or native TF from the first and second species, or other species.

The peptide antigens, immunogens, and antibodies of the present invention can be used in various applications. Among other uses, the peptide antigens can be employed to purify the antibodies as noted above. The synthetic peptides of the present invention can be used to test antibody specificity as noted above. The antigens can also be used in inhibition assays. For example, a peptide from TF can be added to, e.g., thromboplatin assays to determine if it can inhibit TF function. Such work could help characterize TF structure and function.

Applications of antibodies of the present invention include epitope mapping in structure/function studies, immunohistochemical localization, and immunoaffinity purification of the target protein. For example, anti-TF antibodies can be used in a thromboplastin assays to determine if the antibody can inhibit TF function. These antibodies can also be used to study other aspects of TF, such as characteristics of the TF/substrate complex.

The antibodies of the present invention can also be used in diagnostic assays for antigens, particularly where it is required

that the antibodies do not cross-react with antigens from another species (e.g., assays for a particular bacteria in food, etc.)

EXAMPLES

Example 1

Identification of Peptide Immunogens to Rabbit and Human Tissue Factor

The sequences of rabbit, human, and mouse tissue factor were have been described (Pawashe et al., 1991, Thromb Haemost. 66:315). A hydrophilicity analysis was performed using the method of Hopp et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 76:3824. The analysis was performed using the moving averages function on the Excel computer spreadsheet program. Briefly, each amino acid of the rabbit and human sequence was assigned a value from 3.0 to -3.4, where the larger numbers are more hydrophilic. A moving hydrophilicity average of each hexapeptide was established. A mean hydrophilicity average of the hexapeptide averages was then established for regions of the proteins with predominantly positive hexapeptide averages. Regions with mean hexapeptide averages above 0.2 were selected as candidate immunogens for further evaluation. Figures 2 and 3 shows these candidate immunogens.

The candidate immunogens were next evaluated for homology to the mouse sequence. The human and rabbit sequence was aligned with the mouse sequence as in Figure 1. The candidate human and rabbit immunogens were then compared to the aligned mouse sequence and immunogens were selected which have more than 3 nonhomologous amino acids from the analogous mouse sequence.

The selected immunogens are shown as the candidate immunogens of Figures 2 and 3 which have 3 or more mismatches. Eight of the ten candidate immunogens from rabbit TF and eight of the eleven candidate immunogens from human TF. All of those immunogens are useful for producing anti-TF antibodies. However, the most effective immunogens would be those which have a higher mean hydrophilicity value and a larger number of mismatches.

Example 2

Production and Characterization of Polyclonal Antibodies to Rabbit Tissue Factor

Polyclonal antibodies were made to two regions of rabbit TF. The 12 residue peptide TTGFPEEPPFRN from position 84 through 95 (84T-95S), located in the loop connecting domains 1F and 1G and oriented toward the putative top of the molecule (Harlos et al., 1994, Nature 370:662), demonstrated the most amino acids mismatches of the candidate immunogens (Figure 2). The 13 residue peptide VIPSRKRKQRSPE from position 190 through 202 (190V-202E), located within the 2F-2G loop constrained by a disulfide bond, had the highest hydrophilicity index (Figure 2). Both peptides are unique to the rabbit TF sequence when analyzed by the BLASTP search engine with an E value of 100. These two peptides were synthesized using standard FMOC chemistry. An additional cysteine was added to the carboxy terminal. The peptides were then conjugated to MBS-activated KLH by standard methods. For each peptide immunogen, three goats were immunized with the peptide-KLH antigens. Antisera collected from the goats were then tested for immunoreactivity in a microtiter plate format in which unconjugated peptides, rabbit recombinant TF, human recombinant TF, and a Triton extract of rabbit brain acetone powder (RBAP) were the coated antigens. The plates were probed with rabbit anti-goat IgG alkaline phosphatase conjugate.

Each antisera demonstrated specific immunoreactivity to its respective peptide immunogen. All antisera cross-reacted with rabbit recombinant TF. Two antisera, one to each of the peptides, also demonstrated reactivity to natural rabbit TF in the RBAP extracts.

Other features, objects and advantages of the present invention will be apparent to those skilled in the art. The explanations and illustrations presented herein are intended to acquaint others skilled in the art with the invention, its principles, and its practical application. Those skilled in the art may adapt and apply the invention in its numerous forms, as

may be best suited to the requirements of a particular use. Accordingly, the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention.

What is claimed is:

1. A method for selecting a peptide immunogen of a target protein from a first species, wherein the target protein comprises an amino acid sequence which varies between the first species and a second species, the method comprising:
   (a) identifying peptide regions of the amino acid sequence of the target protein from the first species which has a hydrophilicity value greater than 0, wherein the regions are 5-50 amino acids in length; and
   (b) selecting a peptide immunogen from regions identified in step (a) which have at least 1 nonhomologous amino acids between the first species and the second species.

2. The method of claim 1, wherein the hydrophilicity value is greater than 0.2.

3. The method of claim 1, wherein the level of homology between the two species is less than 75%.

4. The method of claim 1, wherein the level of homology between the two species is less than 60%.

5. The method of claim 1, wherein the first and second species are selected from the group consisting of human, rabbit, and mouse.

6. A method of claim 1, wherein the peptide immunogen has at least 3 non-homologous amino acids between the first species and the second species.

7. The method of claim 1, wherein the target protein is tissue factor.

8. The method of claim 7, wherein the target protein is rabbit tissue factor.

9. The method of claim 7, wherein the target protein is human tissue factor.

10. A method of making an antibody which is specific for a target protein of a first species, wherein the target protein comprises an amino acid sequence which varies between the first species and a second species, the method comprising:
(a) selecting a peptide immunogen of the target protein by the method of claim 1;
(b) synthesizing the peptide immunogen;
(c) conjugating the peptide immunogen to an immunogenic carrier molecule to make a peptide-carrier antigen;
(d) producing antibodies to the antigen.

11. The method of claim 10, wherein the peptide antigen is synthesized by recombinant DNA methods.

12. The method of claim 10, wherein the peptide antigen is synthesized using solid phase chemical methods.

13. The method of claim 10, wherein the immunogenic carrier molecule is keyhole limpet hemocyanin.

14. The method of claim 10, wherein the antibodies are monospecific.

15. The method of claim 14, wherein the antibodies are monoclonal antibodies.

16. The method of claim 10, wherein the antibodies are polyclonal.

17. The method of claim 16, wherein the antibodies are made in a mouse, goat, rabbit, or sheep.

18. The method of claim 17, wherein the antibodies are made in a goat.

19. An antibody made by the method of claim 10.

20. A peptide which consists of a sequence from a tissue factor extracellular region from a first species which is 5-50 amino acids in length, has a hydrophilicity value greater than 0, and has at least 1 nonhomologous amino acids from a tissue factor from a second species.

21. The peptide of claim 20, wherein the hydrophilicity value is greater than 0.2.

22. The peptide of claim 20, wherein the homology between the two species is less than 75%.

23. The peptide of claim 20, wherein the homology between the two species is less than 60%.

24. The peptide of claim 20 wherein the peptide has at least 3 non-homologous amino acids from a tissue factor from a second species.

25. The peptide of claim 20, wherein the first species is selected from the group consisting of human, rabbit, and mouse.

26. The peptide of claim 25, wherein the first species is rabbit and the second species is mouse.

27. The peptide of claim 25, wherein the first species is human and the second species is mouse.

28. The peptide of claim 26, selected from the group consisting of 36I-42N, 49L-63K, 84T-95S, 124T-133K, 157R-164K, 174L-179K, 190V-202E, 205T-214R, and 243R-253E.

29. The peptide of claim 28, wherein the peptide is 84T-95S or 190V-202E.

30. The peptide of claim 27, selected from the group consisting of 37Q-44D, 84E-95E, 124N-135R, 158W-169K, 176L-181K, 194P-204D, and 208E-216E.

31. The peptide of claim 30, wherein the peptide is 84E-95E, 124N-135R, 158W-169K, or 194P-204D.

32. An antigen comprising an immunogenic carrier molecule conjugated to the peptide of claim 20.

33. The antigen of claim 32, wherein the carrier molecule is keyhole limpet hemocyanin.

34. An antibody made to the antigen of claim 32.

35. The antibody of claim 34, wherein the antibody is monospecific.

36. The antibody of claim 35, wherein the antibody is a monoclonal antibody.

37. The antibody of claim 34, wherein the antibody is part of a polyclonal antibody preparation.

38. The antibody of claim 37, wherein the antibody is from a mouse, goat, rabbit, or sheep.

39. The antibody of claim 38, wherein the antibody is from a goat.

Figure 1

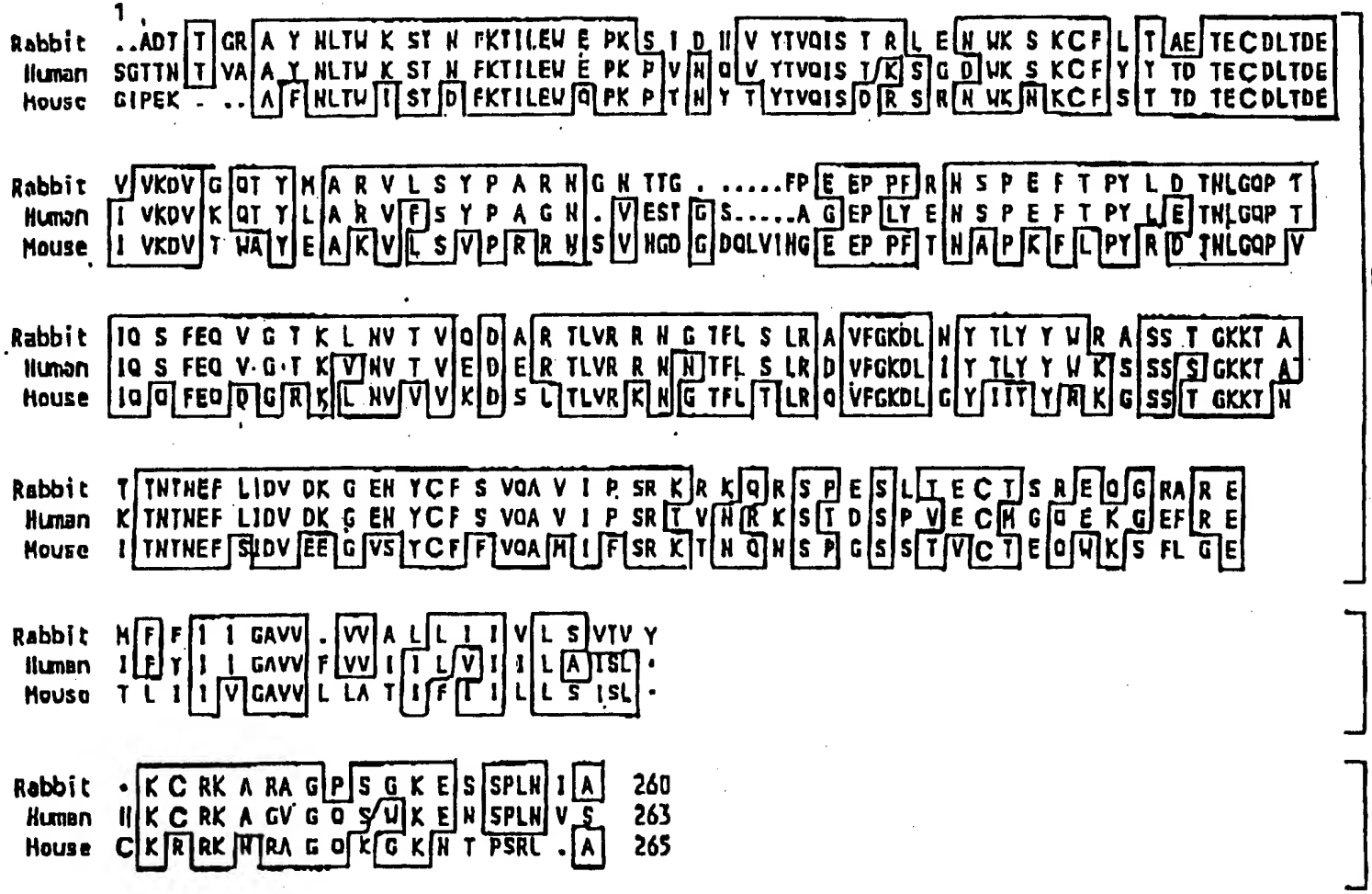

Fig. 1. Homology between rabbit, human, and mouse TF proteins. Identical amino acids are boxed. Gaps (.) are introduced to maximize alignmen Conserved cysteine residues are indicated by bold type

PCT/US00/17609

Figure 2

Rabbit Tissue Factor Hydrophilicity Calculations
(_tp1\ntf-hydrophilicity.xls)

Predicted antigenic determinants by hydrophilicity analysis. Method of T. Hopp & K. Woods, PNAS 78(6): 3824-28, 1981. Moving average of hexapeptides.
Regions of high hydrophilicity analyzed for homology to mouse TF sequence (Pawashe et al, Thrombosis and Hemostasis 66(3): 316-320, 1991) and position in crystal structure of human TF (Harlos et al Nature 370: 662, 1994).
Candidate peptides for immunogens. Best candidate peptides (total length, hydrophilicity, lack of homology, location in molecule) in bold.

Global H mean: mean of the hexapeptide means over this peptide region.
 # mismatches: # of aa positions in rabbit sequence different from mouse sequence in this peptide region.

Peptide Region of TF	Global H mean	# of aa	# mismatches	% homologous	Comments
21L - 26K	0.688	6	1	83%	
36I - 42N	0.493	7	3	57%	
49L - 63K	0.762	15	4	73%	
84T - 95S	0.483	12	5	58%	most dissimilar to mouse. Ms also has an extra 6 aa. located in top loop (connecting loop btwn domain 1 F & G)
124T - 133K	0.495	10	5	50%	
157R - 164K	0.673	8	2	75%	
174L - 179K	1.044	6	3	50%	
180V - 202E	1.310	13	6	54%	very high H value and low homology. Contained within the disulfide looped F&G in domain 2.
205T - 214R	1.185	10	7	30%	contains a cysteine.
243R - 263E	0.820	11	4	64%	10 aa of cytoplasmic domain after last cysteine.

Figure 3

Human Tissue Factor Immunogenic Peptides
(..\tp1\hu ntf-hydrophilicity.xls)

Predicted antigentic determinants by hydrophilicity analysis. Method of T. Hopp & K. Woods, PNAS 78(6): 3824-28, 1981. Moving average of hexapeptides.
Regions of high hydrophilicity analyzed for homology to mouse TF sequence (Pawashe et al, Thrombosis and Hemostasis 68(3): 316-320, 1991) and position in crystal structure of human TF (Harlos et al Nature 370: 662, 1994).
Candidate peptides for immunogens. Bold candidate peptides correspond to 86T-95S and 190V-202E rabbit tissue factor peptides used for immunizations.

Global H mean: mean of the hexapeptide means over this peptide region.
mismatches: # of aa positions in human sequance different from mouse sequence in this peptide region.

Peptide Region of TF	Global H mean	# of aa	# mismatches	% homologous	Comments
23L - 28K	0.486	6	1	83%	22I, 24 E for VII binding
37Q - 44D	0.658	8	4	50%	37Q, (44D, 45W) for VII binding
51Y - 56D	0.799	16	1	94%	58D, 60T for VII binding
84E - 96E	0.250	12	9	25%	most dissimilar to mouse. Ms also has an extra 6 aa. located in top loop (connecting loop btwn domain 1 F & G)
124N - 136R	0.943	12	4	67%	133L, 135R for VII binding
158W - 169K	0.800	12	4	67%	157Y, 158W, 165K, 166K critical residues for X activation
176L - 181K	1.044	6	3	50%	
194P - 204D	0.866	11	7	36%	Contained within the disulfide looped F&G in domain 2.
208E - 216E	1.009	9	6	33%	contains a cysteine.
241S - 247K	0.748	7	2	71%	cytoplasmic domain
251G - 256E	0.5417	6	5	17%	cytoplasmic domain

SEQUENCE LISTING

<110> Goldford, Marc
Triscott, Mark X

<120> SYNTHETIC PEPTIDE IMMUNOGENS AND ANTIBODIES THERETO

<130> sgmd8340.1

<140>
<141>

<150> 60/141,699
<151> 1999-06-30

<160> 11

<170> PatentIn Ver. 2.1

<210> 1
<211> 86
<212> PRT
<213> Rabbit

<400> 1

Ala	Asp	Thr	Thr	Gly	Arg	Ala	Tyr	Asn	Leu	Thr	Trp	Lys	Ser	Thr	Asn
1				5				10						15	
Phe	Lys	Thr	Ile	Leu	Glu	Trp	Glu	Pro	Lys	Ser	Ile	Asp	Ile	Ile	Val
			20					25					30		
Tyr	Thr	Val	Gln	Ile	Ser	Thr	Arg	Leu	Glu	Asn	Trp	Lys	Ser	Lys	Cys
		35					40					45			
Phe	Leu	Thr	Ala	Glu	Thr	Glu	Cys	Asp	Leu	Thr	Asp	Glu	Val	Val	Lys
	50					55					60				
Asp	Val	Gly	Gln	Thr	Tyr	Met	Ala	Arg	Val	Leu	Ser	Tyr	Pro	Ala	Arg
65					70					75					80
Asn	Gly	Asn	Thr	Thr	Gly										
					85										

<210> 2
<211> 141
<212> PRT
<213> Rabbit

<400> 2

Phe	Pro	Glu	Glu	Pro	Pro	Phe	Arg	Asn	Ser	Pro	Glu	Phe	Thr	Pro	Tyr
1				5				10						15	
Leu	Asp	Thr	His	Leu	Gly	Gln	Pro	Thr	Ile	Gln	Ser	Phe	Glu	Gln	Val
			20					25					30		

Gly Thr Lys Leu Asn Val Thr Val Gln Asp Ala Arg Thr Leu Val Arg
35 40 45

Arg Asn Gly Thr Phe Leu Ser Leu Arg Ala Val Phe Gly Lys Asp Leu
50 55 60

Asn Tyr Thr Leu Tyr Tyr Trp Arg Ala Ser Ser Thr Gly Lys Lys Thr
65 70 75 80

Ala Thr Thr Asn Thr Asn Glu Phe Leu Ile Asp Val Asp Lys Gly Glu
85 90 95

His Thr Cys Phe Ser Val Gln Ala Val Ile Pro Ser Arg Lys Arg Lys
100 105 110

Gln Arg Ser Pro Glu Ser Leu Thr Glu Cys Thr Ser Arg Glu Gln Gly
115 120 125

Arg Ala Arg Glu Met Phe Phe Ile Ile Gly Ala Val Val
130 135 140

<210> 3
<211> 14
<212> PRT
<213> Rabbit

<400> 3
Val Val Ala Leu Leu Ile Ile Val Leu Ser Val Thr Val Tyr
1 5 10

<210> 4
<211> 20
<212> PRT
<213> Rabbit

<400> 4
Lys Cys Arg Lys Ala Arg Ala Gly Pro Ser Gly Lys Glu Ser Ser Pro
1 5 10 15

Leu Asn Ile Ala
20

<210> 5
<211> 83
<212> PRT
<213> Human

<400> 5
Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser
1 5 10 15

Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Lys Pro Val His
20 25 30

Gln Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser
35 40 45

Lys Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile
50 55 60

Val Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro
65 70 75 80

Ala Gly Asn

<210> 6
<211> 6
<212> PRT
<213> Human

<400> 6
Val Glu Ser Thr Gly Ser
1 5

<210> 7
<211> 154
<212> PRT
<213> Human

<400> 7
Ala Gly Glu Pro Leu Tyr Glu Asn Ser Pro Glu Phe Thr Pro Tyr Leu
1 5 10 15

Glu Thr Asn Leu Gly Gln Pro Thr Ile Gln Ser Phe Glu Gln Val Gly
20 25 30

Thr Lys Val Asn Val Thr Val Glu Asp Glu Arg Thr Leu Val Arg Arg
35 40 45

Asn Asn Thr Phe Leu Ser Leu Arg Asp Val Phe Gly Lys Asp Leu Ile
50 55 60

Tyr Thr Leu Tyr Tyr Trp Lys Ser Ser Ser Ser Gly Lys Lys Thr Ala
65 70 75 80

Lys Thr Asn Thr Asn Glu Phe Leu Ile Asp Val Asp Lys Gly Glu His
85 90 95

Tyr Cys Phe Ser Val Gln Ala Val Ile Pro Ser Arg Thr Val Asn Arg
100 105 110

Lys Ser Thr Asp Ser Pro Val Glu Cys Met Gly Gln Gln Lys Gly Glu
115 120 125

Phe Arg Glu Ile Phe Tyr Ile Ile Gly Ala Val Val Phe Val Val Ile
130 135 140

Ile Leu Val Ile Ile Leu Ala Ile Ser Leu
145 150

<210> 8
<211> 22
<212> PRT
<213> Human

<400> 8
Ile Ile Lys Cys Arg Lys Ala Gly Val Gly Gln Ser Trp Lys Glu Asn
1 5 10 15

Ser Pro Leu Asn Val Ser
20

<210> 9
<211> 5
<212> PRT
<213> Mouse

<400> 9
Gly Ile Pro Glu Lys
1 5

<210> 10
<211> 240
<212> PRT
<213> Mouse

<400> 10
Ala Phe Asn Leu Thr Trp Ile Ser Thr Asp Phe Lys Thr Ile Leu Glu
1 5 10 15

Trp Gln Pro Lys Pro Thr Asn Tyr Thr Tyr Thr Val Gln Ile Ser Asp
20 25 30

Arg Ser Arg Asn Trp Lys Asn Lys Cys Phe Ser Thr Thr Asp Thr Glu
35 40 45

Cys Asp Leu Thr Asp Glu Ile Val Lys Asp Val Thr Trp Ala Tyr Glu
50 55 60

Ala Lys Val Leu Ser Val Pro Arg Arg Asn Ser Val His Gly Asp Gly
65 70 75 80

Asp Gln Leu Val Ile Asn Gly Glu Glu Pro Pro Phe Thr Asn Ala Pro
85 90 95

Lys Phe Leu Pro Tyr Arg Asp Thr Asn Leu Gly Gln Arg Val Ile Gln
100 105 110

Gln Phe Glu Gln Gln Gly Arg Lys Leu Asn Val Val Val Lys Asp Ser
115 120 125

Leu Thr Leu Val Arg Lys Asn Gly Thr Phe Leu Thr Leu Arg Gln Val
130 135 140

Phe Gly Lys Asp Leu Gly Tyr Ile Ile Thr Tyr Arg Leu Gly Ser Ser
145 150 155 160

Thr Gly Lys Lys Thr Asn Ile Thr Asn Thr Asn Glu Phe Ser Ile Asp
165 170 175

Val Glu Glu Gly Val Ser Tyr Cys Phe Phe Val Gln Ala Met Ile Phe
180 185 190

Ser Arg Lys Thr Asn Gln Asn Ser Pro Gly Ser Ser Thr Val Cys Thr
195 200 205

Glu Asn Trp Ser Lys Phe Leu Gly Glu Thr Leu Ile Ile Val Gly Ala
210 215 220

Val Val Leu Leu Ala Thr Ile Phe Ile Ile Leu Leu Ser Ile Ser Leu
225 230 235 240

<210> 11
<211> 19
<212> PRT
<213> Mouse

<400> 11
Cys Lys Arg Arg Lys Asn Arg Ala Gly Gln Lys Gly Lys Asn Thr Pro
1 5 10 15

Ser Arg Leu

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/17609

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 39/385; C12P 21/00; C07K 16/00, 17/00; G01N 33/48
US CL : 424/193.1, 435/70.21; 530/387.1, 388.1, 391.1; 702/19
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/193.1, 435/70.21; 530/387.1, 388.1, 391.1; 702/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HARLOS et al. Crystal structure of the extracellular region of human tissue factor. NATURE. 25 August 1994, Vol. 370, pages 662-666, see entire article.	7, 9, 20, 25
A	HOPP, T.P. Use of Hydrophilicity Plotting Procedures to Identify Protein Antigenic Segments and Other Interaction Sites. METHODS IN ENZYMOLOGY. 1989, Vol. 178, pages 571-585, see entire article.	1-39

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 SEPTEMBER 2000	Date of mailing of the international search report 18 OCT 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARIANNE DIBRINO Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/17609

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN (EMBASE, BIOSIS, MEDLINE, CAPLUS, SCISEARCH), WEST 2.0
search terms: hydrophilicity, target protein(s), first species, peptide immunogen(s), peptide(s), proteins, tissue factor, Goldford, Marc and Triscott, Mark